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ORIGINAL ARTICLE

Antinociceptive and anti-exudative synergism between dexketoprofen and tramadol in a model of inflammatory pain in mice

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ABSTRACT

Preclinical studies have demonstrated antinociceptive synergism between dexketoprofen (DEX) and tramadol (TRM) in acute animal models of nociception. The aim of the present study was to investigate the type of interaction between DEX and TRM in a chronic musculoskeletal pain model in mice, which fairly replicates the characteristics of chronic osteoarticular pain in humans. Inflammation was induced by a subplantar injection of complete Freund's adjuvant (CFA) in male CF1 mice. Nociceptive thresholds were evaluated using the hot plate, the nocifensive spontaneous behavior and the acetone tests, while plasma extravasation (PE) was assessed with Evan's blue. We used the following experimental groups: control (no inflammation), acute (1 day after CFA injection), and chronic inflammation (7 days after CFA). Dose–response curves for DEX and TRM, individually and combined in a 1 : 1 proportion based on their potency were obtained, and the doses that produced a 50% inhibition calculated. The isobolographic analysis revealed that in all groups of study (no inflammation, acute, and chronic inflammation), the combination of DEX : TRM was synergistic, for both the inhibition of nociception and the PE. The results suggest that the DEX : TRM (1 : 1) combination could be useful in the management of acute and chronic inflammatory musculoskeletal pains in humans; in addition, the synergistic interaction between the drugs observed both during acute and chronic inflammation suggests that less doses would be required of each drug to obtain effective analgesia.

INTRODUCTION

In clinical practice, the combination of analgesics with different mechanisms of action such as opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) is commonly used in the management of different types of acute and chronic pains [1]. When two or more drugs are combined, the pharmacological profile of the combination (beneficial and adverse effects) has to be investigated to establish its potential benefits. However, ethical aspects limit these investigations in humans, and it is also highly impractical, which leads to the use of animal models to establish the optimal drugs and doses to be used in a combination.

Using healthy control mice (without inflammation) and different tests of nociception, preclinical studies performed by our group demonstrated synergy between dexketoprofen (DEX) and TRM when combined in a 1 : 1

proportion based on their potency; the same combination/s showed antagonism on the inhibition of gastrointestinal intestinal transit [2]. Although these results were certainly interesting, they could not be extrapolated to experimental subjects (animal or human) presenting pathological conditions such as acute or chronic inflammatory pain. Furthermore in humans, analgesic drugs (alone or combined) are therapeutically used to treat acute and chronic painful conditions accompanied or not by tissue inflammation.

Thus, the aim of the present investigation was to assess in mice the type of interaction between the DEX and the TRM in a model of inflammatory pain induced by the subplantar (s.pl.) injection of complete Freund's adjuvant (CFA). The model was selected because it mimics some features of chronic musculoskeletal pain in humans and allows the evaluation the antinociceptive and the anti-exudative effects of analgesic drugs individually and combined.

MATERIAL AND METHODS

Animals

In all experiments, CF1 male mice weighting 28–30 g were used; during the study, they were housed under a 12-h light/dark cycle at 22 °C, with free access to food and water. The protocol was approved by the Bioethics Committee of Animal Research, Faculty of Medicine of the University of Chile and the Ethical Committee for Animal Welfare of the Institution PRBB, Barcelona, Spain, according to the rules of the International Association for the Study of Pain. Animals were acclimated to the conditions in the animal facilities for at least 7 days before the experiments. Mice were used only once for a particular test or protocol, and at the end of the experiment they were sacrificed by cervical dislocation. Before starting the experiments, animals were randomly distributed in boxes according to the study protocol, and the investigator performing the test was blinded to the administration of drugs and doses; however, because of the characteristics of the study, the presence of inflammation of the paw was easily identifiable by the researcher conducting the experiment and the researcher could not be blinded.

Induction of inflammation

Animals received a s.pl. injection of 30 μL CFA in the right hindpaw according to the method described by Larson et al. [3]. At this dose, CFA induces an inflammatory reaction restricted to the injected paw, which persists for a period of 14 days [4]. Noninjected animals were used as control, because the s.pl. injection of the same volume of saline into the paw induces a mild but statistically significant inflammatory reaction that could interfere with the evaluation of the results [5]. Thus, the experiments were performed in mice without inflammation and in mice 1 day (acute inflammation) and 7 days (chronic inflammation) after the injection of CFA.

Evaluation of thermal hyperalgesia by the hot plate test

We used a modification of the method previously described by Melendez et al. [6] where the animals can move freely during the experiment. The temperature of the hot plate (HP) was kept constant at 45 ± 1 °C by a digitally thermoregulated circulating pump. The animals were placed on the HP, and the latency of onset of signs of nociception (licking of one or both forepaws and/or jumping from the hot surface) was recorded by a stopwatch. The maximum basal latency or time of cut (cutoff) was set at 30 s to prevent tissue damage. With an interval of 3 min, we took two measurements at baseline and two after the administration of study drug; the resulting averages in latency were taken as basal and experimental, respectively. Approximately 3% of the animals had basal latencies lower than 25 s and were eliminated from the study. Latency times were transformed into % of maximum possible effect (MPE or % antinociception) according to the following expression: $\%\text{MPE} = 100 \times [(L_2 - L_1)/(T_1 - L_1)]$, where L_1 = baseline latency, L_2 = postdrug latency, T_1 = cutoff time.

Evaluation of spontaneous behavior with the nocifensive assay

After 10 min of habituation to the environment, the animals were placed in a plastic box, and signs of spontaneous pain were recorded during a period of 10 min (600 s) [7]. The nociceptive score was assigned according to the method described by Dubuisson and Dennis [8] for the formalin test: a value 0 is assigned when inflamed paw (that had been injected with CFA) is in normal contact with the floor of the box; stage 1 if the contact with the floor is light and the body weight rests on the opposite paw; stage 2 when there is no contact of the paw with the floor of the box; and stage 3 when there is no contact with the floor and the animal licks the inflamed paw.

The final score is obtained by multiplying the time in seconds that the animal remains at each stage, by the value assigned (1, 2, 3), divided by observation time in

seconds, according to the following expression: $(T_1 \times 1) + (T_2 \times 2) + (T_3 \times 3)/600$, where T_1 = time in stage 1, T_2 = time in stage 2, T_3 = time in stage 3, 600 = total observation time.

Evaluation of cold hyperalgesia

We used the acetone test (AT) according the method described by Smith et al. [9], which consists of applying 20 μL of acetone to the plantar surface of both hindpaws (inflamed and contralateral). From the time of application onwards, the time (in seconds) that the animal licks or shakes the paw is recorded. The duration of the response was recorded during a maximum time of 20 s [10,11]. For each paw, two measurements were taken separated by an interval of 3 min, and the mean value obtained. We also measured basal reaction time in untreated control animals to compare the results with those obtained in the contralateral paw.

Evaluation of plasma extravasation (anti-exudative effect)

A modification of the method described by Emanueli et al. [12] was used. Animals were lightly anesthetized with sevoflurane and were injected into the retroorbital plexus 50 mg/kg of Evans blue dye (EBD), a marker of extravascular protein leakage, in 0.085 mL in saline solution. When injected intravenously, the EBD binds to plasma proteins and thus remains within the vasculature. In the presence of inflammation, plasma extravasation (PE) leaks out into the tissues and can be used as a marker for PE. Fifteen minutes after PE injection, animals were sacrificed by cervical dislocation, and both hindpaws (inflamed and noninflamed) severed, weighed, and cut into sections. Then, they were incubated with 1 mL of formamide at room temperature for 48 h to extract the PE dye. The concentration of dye in the supernatant fluid was measured by spectrophotometry at 620 nm (SmartSpec3000; BioRad, Hercules, CA, USA). Absorbance was calculated from a standard calibration curve (0.05–25 μg/mL of albumin in formamide and results expressed as absorbance units (AU) per gram of wet tissue. To determine PE, the concentration of PE in the contralateral paw was substracted from the value obtained in the inflamed paw, as no significant extravasation of PE was detected in control mice (no inflammation). The inhibitory effects drugs have on plasma extravastion were determined according to the following equation: %MPE = [(AU paw inflamed predrug − AU paw inflamed postdrug)/(AU paw inflamed predrug)] × 100.

Assessment of the type of interaction between DEX and TRM

The type of interaction was evaluated with an isobolographic analysis of the combination of DEX with TRM, in agreement with the method previously described [13,14]. For each test, dose–response curves for DEX and TRM, each one individually, were obtained, using at least four doses per curve and 6–8 animals per dose. These experiments were conducted in the acute inflammatory (1 day after CFA) and chronic phase (7 days post-CFA). From the corresponding dose–response curves and by least-square linear regression analysis, the dose that produces a 50% MPE (ED_{50}s) of each drug was calculated. Then, additional dose–response curves were generated with the combination of the two drugs combined in a 1 : 1 ratio, based on their analgesic potency (ED_{50}). In this study, the ED_{50} is defined as the dose of a drug or a combination of drugs, which produces a 50% of the MPE. The ED_{50}s obtained experimentally were compared with the theoretic ED_{50}s obtained on the basis of the assumption that the effects of the drugs were additive (no interaction).

For each test (nociceptive behaviors, PE) and experimental condition (acute or chronic inflammation), isobolograms and interaction indexes (I.I.) were obtained according to the methods previously described [13,14].

Drugs

The antinociceptive and anti-exudative effects of DEX and the TRM (individually and in combination) were evaluated after 30 min of administration of the drugs, time of peak efficacy for these drugs [13,14] which were administered intraperitoneally (i.p.) in a constant volume of 10 mL/kg. Control animals received the same volume of saline.

Statistical analysis

The results are presented as mean values ± SEM or 95% confidence limits. Isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27; the McCary Group Inc., Philadelphia, PA, USA) based on Tallarida 2000 [15]. The data were analyzed by Student's *t*-test or one-way ANOVA, followed by the Student–Newman–Keuls test. P values <0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

Evaluation of heat hyperalgesia in the hot plate test

In this test, the basal latency before CFA inflammation was 19.05 ± 0.98 s; this value significantly decreased

on day 1 after CFA (CFA-1, acute inflammation) to 13.1 ± 0.80 s, further decreasing to 10.5 ± 0.60 s on day 7 (CFA-7, chronic inflammation). The values obtained on days 1 and 7 post-CFA were significantly different to both control and baseline values ($P < 0.05$), and also when compared each other ($P < 0.05$). In control animals without inflammation, the administration of increasing doses of DEX (range 30–250 mg/kg, i.p.) or TRM (10–100 mg/kg, i.p.) induced dose-dependent antinociception in the HP test, with ED_{50}s of 120.5 ± 9.4 mg/kg and 25.2 ± 1.3, respectively (*Table I*). The slopes of the DEX and TRM curves were 81.47 ± 9.06 and 75.20 ± 4.68 ($P < 0.05$), demonstrating that the dose–response curves were parallel (*Figure 1a*). When both drugs were combined (1 : 1 proportion), the ED_{50} value of the resulting curve was 59.60 ± 1.28 mg/kg (*Table II*).

In the presence of inflammation, dose–response curves were also obtained for DEX and TRM individually, 1 (*Figure 1b*) and 7 days (*Figure 1c*) after CFA injection. The ED_{50} values for DEX were: 109.1 ± 12.85 and 94.1 ± 9.37 mg/kg, on days 1 and day 7, respectively (*Table I*); the ED_{50}s for TRM were 22.71 ± 1.53 and 19.31 ± 1.47 mg/kg, on days 1 and 7 after CFA administration (*Table II*). The results show that acute inflammation (CFA-1) did not modify the potencies of DEX and TRM (when compared to control). Only during chronic inflammation (CFA-7), the potencies of DEX and TRM were significantly increased (Student's *t*-test, $P < 0.05$) when compared to control.

Table I ED_{50}s (mg/kg ± SEM) for dexketoprofen (DEX) and tramadol (TRM) given alone and potency ratio, in the hot plate (HP), the spontaneous nocifensive behavior (SN), the acetone (A) tests, and on plasma extravasation (PE).

Treatment test	Control ED_{50}	CFA-1 day (acute inflammation) ED_{50}	CFA-7 days (chronic inflammation) ED_{50}
DEX			
HP	120.50 ± 9.4	109.10 ± 12.85	94.10 ± 9.37*
SN	11.80 ± 0.84	8.65 ± 0.41*	9.30 ± 0.24*
AA	6.00 ± 0.38	8.65 ± 0.81*	10.60 ± 0.25*,**
PE	–	13.60 ± 1.26	16.35 ± 0.35
TRM			
HP	25.20 ± 1.3	22.71 ± 1.53	19.31 ± 1.47*
SN	6.36 ± 0.25	6.41 ± 0.41	5.90 ± 0.24
AA	4.90 ± 0.14	5.00 ± 0.28	6.10 ± 0.14*,**
PE	–	2.90 ± 0.40	7.09 ± 0.70**
Relative potency (DEX/TRM)			
HP	4.78	4.80	4.80
SN	2.00	1.30	1.60
AA	1.20	1.70	1.70
PE	–	4.60	2.30

AA, acetone allodynia; CFA, complete Freund's adjuvant; ED_{50}s; dose that produces a 50% MPE.

Results obtained in animals without inflammation (control), or during acute (CFA-1 day) and chronic inflammation (CFA-7 days). Drugs were administrated intraperitoneally (i.p.) *indicates $P < 0.05$ comparing vs. control, and **$P < 0.05$ compared to CFA-1 day.

The combination of DEX and TRM (1 : 1 proportion) also generated dose–response curves during acute and chronic inflammation, with ED_{50} values of 29.91 ± 0.71 and 21.27 ± 1.25 mg/kg, respectively (*Table II*). Both ED_{50}s were significantly lower when compared to control animals (Student's *t*-test, $P < 0.05$), and they were also different among themselves (Student's *t*-test, $P < 0.05$). Thus in the HP test, the potencies of DEX, TRM, and their 1 : 1 combination, significantly increased during chronic inflammation.

The I.I. and the isobolographic analysis at the 50% level of effect showed synergy between DEX and TRM in control animals (*Table II*, *Figure 1d*) and in the presence of acute (*Figure 1e*) and chronic inflammation (*Figure 1f*). The I.I. were 0.292 for control animals, and 0.625 and 0.186 for animals with acute and chronic inflammation, respectively (*Table II*). It is interesting to note that the magnitude of synergy was higher in the presence of chronic inflammation.

Evaluation of spontaneous nocifensive (SN) behavior

In control animals, the administration of increasing doses of DEX (range 3–20 mg/kg, i.p.) or TRM (1–15 mg/kg, i.p.) induced dose-dependent antinociception, with ED_{50} values of 11.8 ± 0.84 and 6.36 ± 0.25 mg/kg, respectively (*Table I*). During acute inflammation, the ED_{50}s were 8.65 ± 0.41 mg/kg for DEX and 6.41 ± 0.41 mg/kg for TRM (*Table I*), while during chronic inflammation, the ED_{50}s were 9.30 ± 0.24 mg/kg for DEX and 5.90 ± 0.24 mg/kg for TRM (*Table I*). The potency of DEX but not that of TRM was significantly higher during inflammation (Student's *t*-test, $P < 0.05$) when compared to controls, but no differences were observed between acute and chronic inflammation.

The coadministration of DEX and TRM (1 : 1 proportion) produced dose–response curves in all experimental conditions, with ED_{50} values of 9.08 ± 0.43, 3.10 ± 0.01, and 4.20 ± 0.01 mg/kg for control animals, and during acute and chronic inflammation, respectively (*Table II*). The ED_{50}s of the combination obtained during inflammation were significantly lower than in the control animals, but no differences could be

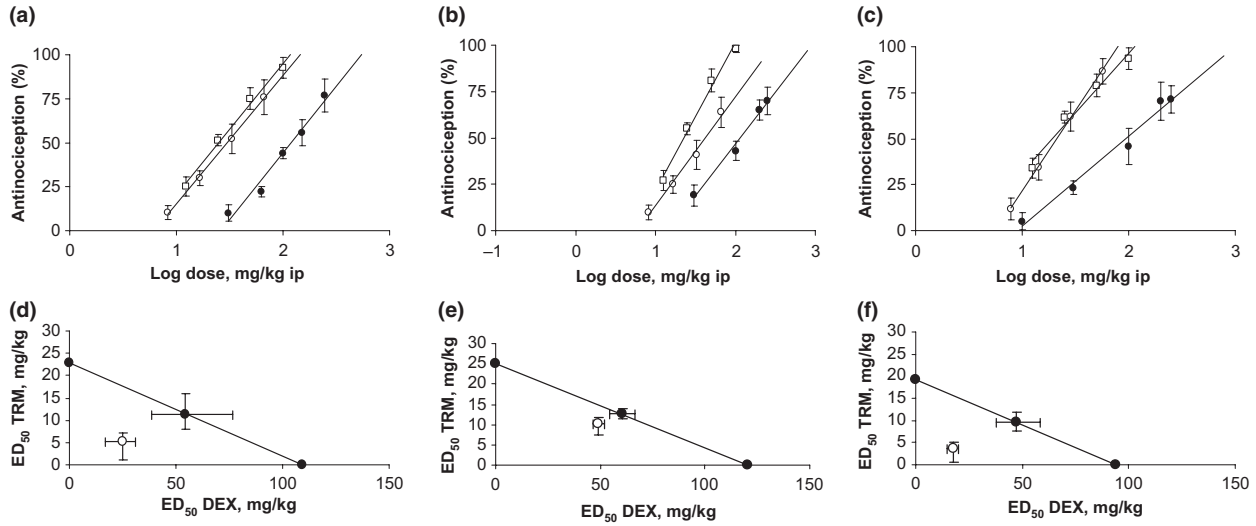

Figure 1 Dose–response curves for the antinociceptive effects of intraperitoneal dexketoprofen (DEX) (•) or tramadol (TRM) (□) alone and combined (○) in a 1 : 1 ratio, in the hot plate. Panel a: control animals without inflammation; panel b: 1 day after complete Freund's adjuvant (CFA); panel c: 7 days after inflammation. Panel d: isobologram DEX : TRM (1 : 1) at the dose that produces a 50% MPE (ED_{50}) in control animals; panel e: isobologram 1 day after CFA; panel f: 7 day after inflammation. Filled circles indicate the theoretic ED_{50s} with 95% confidence limits (CL); open circles are the experimental ED_{50s} with 95% CL. In panels a, b, and c, each point represent the mean values of 6–8 animals with the SEM. In panels d, e, and f. Each point represents the mean value of eight experiments and vertical and horizontal lines corresponding to CL.

Table II ED_{50}s (mg/kg ± SEM), interaction indexes (I.I.) and type of interaction for the 1 : 1 combination of dexketoprofen (DEX) and tramadol (TRM), in the hot plate (HP), the spontaneous nocifensive behavior (SN), the acetone (A) tests, and on plasma extravasation (PE).

Treatment	Control			CFA-1 day (acute inflammation)			CFA-7 days (chronic inflammation)		
Test	ED_{50}	I.I.	Interaction	ED_{50}	I.I.	Interaction	ED	I.I.	Interaction
DEX : TRM									
HP	59.60 ± 1.28	0.292	Synergy	29.91 ± 0.71*	0.625	Synergy	21.27 ± 1.25*,**	0.186	Synergy
SN	9.08 ± 0.43	0.430	Synergy	7.53 ± 0.03*	0.475	Synergy	7.60 ± 0.01*	0.553	Synergy
AA	5.15 ± 0.12	0.716	Synergy	6.82 ± 0.03	0.308	Synergy	8.35 ± 0.10*,**	0.371	Synergy
PE	–			5.63 ± 1.35	0.67	Additive	1.34 ± 0.17**	0.11	Synergy

AA, acetone allodynia; CFA, complete Freund's adjuvant; ED_{50}s; dose that produces a 50% MPE.

Results obtained in animals without inflammation (control), and during acute (CFA-1 day) and chronic inflammation (CFA-7 days). Combinations were DEX : TRM = combination of the ED_{50} of DEX plus the ED_{50} of tramadol, for each nociceptive test and experimental condition. Drugs were administrated intraperitoneally (i.p.) *indicates $P < 0.05$ comparing vs. control, and **$P < 0.05$ compared to CFA-1 day. Lower values of I.I. indicate higher potency of the drug combinations.

established between acute and chronic inflammation (comparing vs. acute, Student's *t*-test, $P < 0.05$).

The I.I. values obtained with the combination of DEX and TRM were of 0.430 in control animals, and 0.475 and 0.553 for animals with acute and chronic inflammation, respectively, demonstrating synergy (*Table II*). In *Figure 2*, we show the isobolographic representation of the results at 50% level of effect (*Figure 2a*, *b*, and *c*). The magnitude of synergy was similar in the three experimental conditions.

Evaluation of cold thresholds using the acetone (AT) test

Nociceptive thresholds to cold (referred in this report as *acetone allodynia*, AA) in baseline conditions was 3.08 ± 0.27 s. The administration of increasing doses of DEX (ranging from 0.50 to 20 mg/kg, i.p.) or TRM (1–15 mg/kg, i.p.) induced dose-dependent antinociceptive effects in control animals (no inflammation) and also during acute and chronic inflammation. ED_{50} values in control mice were 6.0 ± 0.38 and 4.9 ± 0.14 mg/kg for

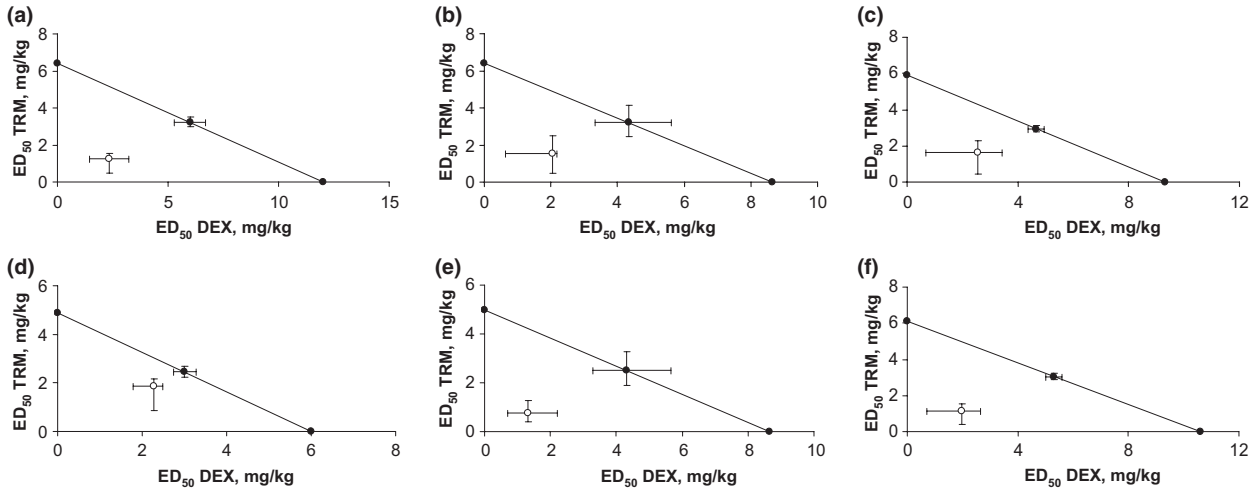

Figure 2 Isobologram of the antinociceptive effects of the combination dexketoprofen : tramadol at the dose that produces a 50% MPE (ED_{50s}) in the spontaneous nocifensive behaviors. Panel a: isobologram in control animals; panel b: isobologram 1 day after complete Freund's adjuvant (CFA); panel c: 7 day after inflammation. Isobologram of the nociceptive activity in cold allodynia. Panel d: isobologram in control animals; panel e: isobologram 1 day after CFA; panel f: 7 day after inflammation. Filled circles indicate the theoretic ED_{50s} with 95% confidence limits (CL); open circles are the experimental ED_{50s} with 95% CL. Each point represents the mean value of eight experiments and vertical and horizontal lines corresponding to CL.

DEX and TRM, respectively (*Table I*). For DEX, acute inflammation significantly increased the ED_{50} to 8.65 ± 0.81 mg/kg ($P < 0.05$), which was slightly but significantly higher during chronic inflammation (10.6 ± 0.25 mg/kg, $P < 0.05$ when compared acute inflammation). However, the potency of TRM was similar to control during acute inflammation (ED_{50} value 5.0 ± 0.28 mg/kg) but significantly increased to 6.1 ± 0.14 mg/kg during chronic inflammation (*Table I*).

The coadministration of DEX and TRM (1 : 1 proportion) produced dose–response curves in all experimental conditions, with ED_{50}s of 5.15 ± 0.12, 2.10 ± 0.03, and 3.10 ± 0.01 mg/kg for mice in the control, acute, and chronic inflammation groups, respectively (*Table II*). Antinociceptive potencies obtained during inflammation were significantly different from control animals (Student's *t*-test, $P < 0.05$).

The analysis of the interaction demonstrated synergy at the 50% level of effect in all experimental conditions (*Table II* and *Figure 2*) The I.I. values obtained with the combination of DEX and TRM were of 0.716 for control animals, and 0.308 and 0.371 for animals with acute and chronic inflammation, respectively, demonstrating synergy. Similarly, the isobolographic representation of the results also showed synergy of a similar magnitude during acute and chronic inflammation (*Figure 2d, e*, and *f*).

When comparing the antinociceptive potencies obtained from the different tests used in the evaluation of nociceptive behavior, TRM was between 1.2 (AA) and 4.8 times (HP) more potent than DEX, a potency ratio that was maintained in the three experimental conditions (*Table I*).

Evaluation of plasma extravasation

In control mice (no inflammation), basal protein leakage was 1.10 ± 0.20 AU/g, which was significantly increased during acute and chronic inflammation to 4.27 ± 1.44 and 1.90 ± 0.40 AU/g, respectively ($P < 0.05$ when compared to controls). Thus, a higher PE was observed 1 day after CFA injection (acute inflammation).

Dose–response relationships were established for each drug individually and combined in a 1 : 1 proportion in untreated animals (control) and in the presence of acute (1 day) and chronic (7 days) inflammation (*Figure 3*). The resulting dose–response curves had calculated E_{max} values between 60 and 70% (*Figure 3a* and *b*). The ED_{50} values during acute inflammation were 13.6 ± 1.26 mg/kg for DEX, 2.901 ± 0.40 mg/kg for TRM (*Table I*), while the DEX : TRM combination had an ED_{50} of 5.63 ± 1.35 mg/kg (*Table II*). The ED_{50} values for CFA-7 days condition were 16.35 ± 0.35 mg/kg for DEX, 7.09 ± 0.7 mg/kg for TRM (*Table I*), and 1.34 ± 0.17 mg/kg for the combination DEX : TRM (*Table II*).

The results indicate that in our experimental conditions, the anti-exudative effects of TRM are approximately

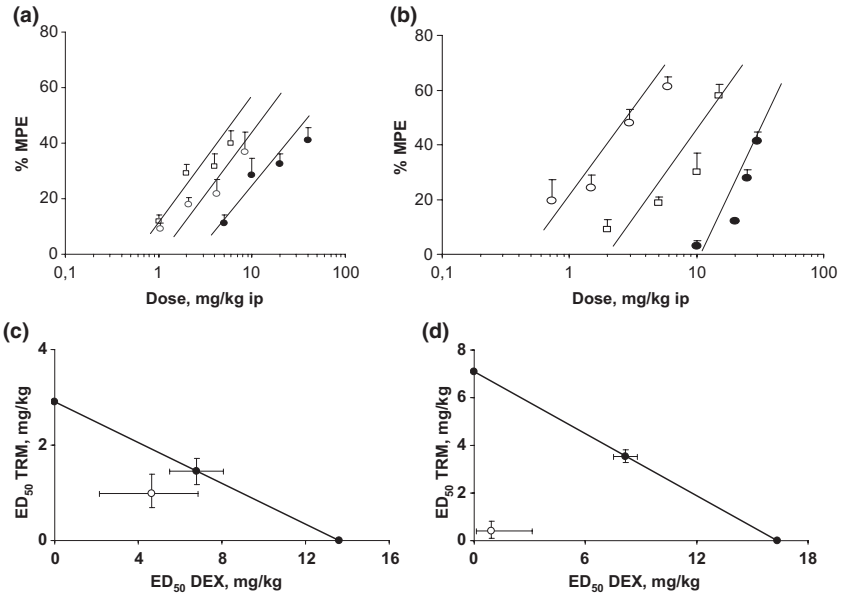

Figure 3 Dose–response curves for the inhibition of plasma extravasation of dexketoprofen (DEX) (●) or tramadol (TRM) (□). alone and combined (○) in a 1 : 1 potency ratio (panels a and c). Isobologram of the antinociceptive effects of the combination DEX : TRM at the dose that produces a 50% MPE (ED_{50s}) (panel c and d). Panels a and b represent the dose–response curves for each individually and in combination, at complete Freund's adjuvant (CFA) 1 and 7 days, respectively. The results are expressed as MPE, and each point is the mean of 12–14 animals and vertical bars indicate SEM. Panels c and d: isobologram 1 day after CFA; panel f: 7 day after inflammation. Filled circles indicate the theoretic ED_{50s} with 95% confidence limits (CL); open circles are the experimental ED_{50s} with 95% CL. Each point represents the mean value of eight experiments and vertical and horizontal lines corresponding to CL.

4.5 and 2.0 times higher than those of DEX during acute and chronic inflammation, respectively (*Table I*). Moreover, while DEX shows similar potency during acute and chronic inflammation ($P = 0.049$), the potency of TRM is significantly higher during acute inflammation ($P < 0.05$) (*Table I*).

The I.I. and isobolographic analysis of the DEX : TRM combination (1 : 1) at the 50% level of effect demonstrated that the effects were additive (I.I. = 0.67) during acute inflammation but became strongly synergistic (I.I. = 0.11) 7 days after CFA administration (*Table II* and *Figure 3c* and *d*).

DISCUSSION

In a mice model of musculoskeletal pain, we have investigated the antinociceptive and anti-exudative effects of DEX and TRM individually, and combined in a 1 : 1 proportion. The drugs and their combinations induced dose-related inhibitions in the HP, SN, and ATs, as well as on PE. The I.I. and isobolographic analysis demonstrated synergy at the 50% level of effect in all tests. To our understanding, this is the first report demonstrating synergy between an NSAID and a weak opioid on the inhibition of PE during chronic inflammation. Globally, the results indicate that lower doses of each individual drug are required to induce the same degree of analgesia/anti-extravasation, and as a consequence a decrease in drug-related toxicity could be anticipated.

In mice, the s.pl. injection of CFA induces a local long-lasting inflammatory response [3], associated with peripheral and central sensitization and increased PE; the response has been previously characterized by our group [4] and can be observed from 4 h to 14 days after CFA injection. In the present report, we used 1 and 7 days to investigate the effects of DEX and TRM during acute and chronic inflammation, respectively. It is interesting to note that independent of the behavioral test and experimental condition (no inflammation, acute or chronic inflammation) assessed, the anti-exudative and antinociceptive and potency of TRM was higher than that of DEX. The latter was previously reported by our group in acute tonic nociceptive models of nociception (induced by chemical and heat stimuli) [2].

Thus, the present results confirm that opioids and NSAIDS show different profiles of nociceptive activity,

independent of the type of nociceptive stimulus or the type of pain (acute or chronic).

It has been described that paw inflammation (induced by carrageenan, capsaicin, CFA, etc.) increases the potency of opioids after local or systemic administration [16,17]. However in our study, the antinociceptive potency of tramadol was only slightly increased during chronic inflammation after thermal nociceptive stimulus, but not in the SN or ATs. Interestingly, the ED_{50}s obtained after the SN behavior, and ATs were significantly lower than those obtained in the HP, suggesting that different nociceptive mechanisms participate in the analgesia induced by tramadol. In the evaluation of SN behavior, no differences were observed in the ED_{50}s in the three experimental conditions, whereas cold stimuli induced a significant and unexpected decrease in the potency of both analgesics (DEX and TRM) mainly during chronic inflammation (*Table I*). At present, we have no sound explanation for the increase in the ED_{50}s of both analgesics in front of a cold stimulus (AT). The potency of DEX significantly increased during chronic inflammation in the HP and SN tests. Mazario et al. [18] reported that intravenous DEX was 40 times less potent in rats with paw inflammation compared with control animals after mechanical stimulation (Von Frey filaments). These differences could be related not only to the species and route of drug administration used, but mainly to the type of stimuli applied. Electrophysiological studies show that most C-fiber nociceptors are polymodal and can be activated by multiple types of stimuli. Cain et al. [19] reported that heat stimuli evoked responses from the majority of C-fibers (88%), 77% of C-fibers were excited by cold, and 68% were excited by both stimuli, although these values depended on the stimulus intensity. Taken together, our results show the importance of the nature of the type of noxious stimulus used for evaluating therapeutic effects of drugs. It may be noted that recent studies [20] have identified two cold-activated transient receptor potential (TRP) channels present in sensory neurons as transducers of cold stimuli. TRPM8 seems to mediate responses to cooling while TRPA1 is activated, possibly indirectly, by more extreme cold conditions. However, the existence of cold-responsive neurons that do not express these channels suggests that other transducers of cold stimuli remain to be discovered. Subsequent action potential electrogenesis and probably propagation from sensory neurons innervating cold nociceptive neurons, plays an important role in pain pathway. This may be an interpretation why in front of a cold stimulus (acetone), we need higher doses of analgesics. In addition, transient receptor potential A1 (TRPA1) forms nonselective cation channels and are implicated in acute inflammatory pain and nociception. NSAIDs are structural analogs of prostaglandins (PGs) and can act as TRPA1 agonists. Thus, extracellularly applied flufenamic, niflumic, and mefenamic acid, as well as flurbiprofen, ketoprofen, diclofenac, and indomethacin, rapidly activated rat TRPA1 expressed in Xenopus oocytes and human TRPA1 endogenously expressed in WI-38 fibroblasts. Similarly, the NSAID ligands activated human TRPA1 inducibly expressed in HEK293 cells [21]. They are the TRP proteins, which serve as transmembrane pathways for Ca (2+) influx and cyclo-oxygenase 1 (COX-1), a key enzyme in the formation of PGs [22]. This findings could be applied to the involvement of TRP channels in the action of DEX and TRM.

Moreover, when comparing the ED_{50}s of DEX and TRM individually obtained in the present investigation with those obtained in other nociceptive assays [2,23], a significant decrease in the antinociceptive potency of both drugs can be observed. This could indicate that not only the type of stimulus but also the type of pathological situation (acute or chronic inflammation) may determine the drug effects.

In our study, we also evaluated the anti-exudative effects of DEX, TRM, and their 1 : 1 combination. Inflammatory mediators such as bradykinin, 5-hydroxytryptamine (5-HT), histamine, ATP, PGs, and cytokines are released in the periphery upon noxious stimulation or injury, causing nociception and PE [24]. In our chronic pain model, the presence of peripheral inflammation induced a significant increase in PE in the inflamed hindpaw. Extravasation was higher during acute than chronic inflammation (1 day), an observation that has been previously reported by our group [4]. Although each drug individually induced dose–response relationships for extravasation, the TRM curves reached a maximal inhibition in the range of 60–80% during acute and chronic inflammation, respectively. The expression of MOR receptors (MOR) in endothelial cells and their involvement in the mechanisms involved in PE have been previously reported by others [25]. We have also reported that pure MOR agonists such as morphine or fentanyl induced a 100% inhibition of PE during acute inflammation in rats [26]. The low affinity of TRM for MOR and its dual mechanism of action could explain the low Emax values observed.

Nonsteroidal anti-inflammatory drugs, such as DEX, induce their pharmacological effects inhibiting the nonspecific COX with the consequent decrease in PGs

synthesis [27]. We did not observe significant differences in the anti-exudative effects of DEX during acute or chronic inflammation, indicating that in the present experimental conditions, the efficacy of this drug is independently of the magnitude of exudation.

It is widely accepted that the concurrent administration of two or more drugs in different experimental conditions can alter pharmacokinetics and/or pharmacodynamics, modifying the pharmacological profile. In general, synergism requires that the drugs combined have different mechanism [28]. In our work, we combined DEX, a nonselective COX inhibitor, [18] and TRM, an atypical centrally acting analgesic with opioid and monoaminergic mechanisms of action, and were able to demonstrate synergy on antinociception and inhibition of extravasation, the latter more evident during chronic inflammation [29]. Other studies have shown synergistic interactions for antinociception between NSAIDs and adrenergic drugs [30–32]; however, to or knowledge, the interaction between NSAIDs and opioids on extravasation has not been previously reported. Taken together, the present findings suggest that the use of DEX combined with tramadol would improve its therapeutic profile, especially in the presence of chronic inflammation.

The interaction between DEX and TRM could occur in the central nervous system and/or peripherally, where COX isoforms, opioids and α-aminergic receptors, involved in the processing of nociceptive and exudative responses have been identified. It has been suggested [33] that in the spinal cord, PGs may inhibit presynaptic noradrenaline (NA) release. Consequently, the inhibition of PGs synthesis induced by DEX would increase NA levels, and the results overlap with the reuptake inhibition of NA induced by tramadol.

When compared with the administration of strong opioids, it is well accepted that tramadol induces analgesia with negligible organ toxicity and offers some benefits in the management of musculoskeletal and neuropathic pains [34]. Moreover, current guidelines recommend avoiding or reducing NSAIDs for long-term pain management because of their risks in the gastrointestinal and cardiovascular systems [35]. The present findings show that it is possible to obtain effective analgesia by the simultaneous administration of DEX and TRM because of their synergistic interaction [1]. It is likely that the activation of different endogenous antinociceptive pathways by the combination may lie at the basis of the synergistic effects of the combinations [36].

In conclusion, our data show synergy for the antinociceptive and anti-exudative effects of the 1 : 1 combination of DEX and tramadol in a murine model of chronic inflammation, suggesting that the mixture could be advantageous in the management of musculoskeletal pain in human. The synergistic interaction in the inhibition of PE is remarkable and has important therapeutic implications. Specifically, it challenges the artificial division of drugs for arthritis between those that manage symptoms vs. the disease-modifying therapies. If analgesics such as tramadol can synergize with NSAIDS to reduce inflammation, perhaps long-term they can attenuate the progressive bone and joint degradation associated with arthritic conditions.

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ABBREVIATIONS

DEX – dexketoprofen
TRM – tramadol
CFA – Complete Freund's Adjuvant
MPE – maximum possible effect
EBD – Evans blue dye
CL – confidence limits
s.pl. – subplantar
HP – hot-plate
SN – spontaneous nocifensive behavior
AT – acetone test
AA – acetone allodynia
PE – plasma extravasation
AU – absorbance units
ED_{50} – dose that produces a 50% MPE
NSAIDs – nonsteroidal anti-inflammatory drugs
PGs – prostaglandins

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